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PREDOMINANT VARIABLE REGION GENE USAGE BY γ/δ T CELL RECEPTOR-BEARING CELLS IN THE ADULT THYMUS

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Most T lymphocytes express a clonally distributed cell surface antigen receptor composed of disulfide-linked α and β subunits, noncovalently associated with several subunits called CD3 proteins. T cells that express the α/β form of the T cell receptor (TCR) recognize antigens in the context of cell surface proteins encoded by class I and class II genes of the major histocompatibility complex (MHC). A small distinct subset of peripheral T lymphocytes (<5%) and thymocytes expresses an alternative CD3-associated receptor composed of disulfide-linked γ and δ subunits; most of these so-called γ/δ cells are also distinguished from most α/β -expressing T cells by their CD4⁻, CD8⁻ (double-negative) phenotype (1-7). The function and specificity of γ/δ receptors and the cells that express them, while unknown, is of great interest, because they may mediate a previously unappreciated immune function and/or represent an intermediate stage in T cell development. A crucial issue in evaluating the functional capabilities of these cells concerns the potential diversity of ligands that can be recognized by the γ/δ receptor. Although these ligands have not been identified, their potential diversity should be related to the expressed diversity of the γ/δ receptor itself. We have begun to analyze the diversity of γ and δ chains used by T cells specifically selected for expression of a γ/δ heterodimer on their surface. This approach also allows us to assess the coexpression of particular V γ and V δ chain pairs by these cells. This approach contrasts that of isolation of γ and δ cDNA clones from populations of cells in which many cells do not express a γ/δ T cell receptor, and where isolation of in-frame cDNAs does not necessarily imply functional expression.

Like other T cell receptor and immunoglobulin subunits, the γ subunit is encoded by distinct variable (V), joining (J), and constant (C) region gene segments that have characteristic similarities to immunoglobulin amino acid sequence and undergo

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somatic rearrangements (of V to J) to generate a complete functional gene (8–11). In the mouse, three functional C genes, each with an associated J segment, and seven potentially functional V genes have been detected (Fig. 4 A) (9–13). Therefore, the potential V-region diversity of the γ chain is limited compared with that of the immunoglobulin heavy or light chains, and the α and β subunits of the α/β TCR.

Recently, a fourth rearranging TCR-like gene, called “x”, was isolated by Chien et al. (14). More recently, serological evidence (15–18), and amino acid sequence analysis of a δ chain from a hybridoma (19), have conclusively shown that the x gene encodes the δ chain. The C δ gene is located just 5' to the known J α gene segments in the mouse, and is therefore embedded between the V α gene segments and the J α -C α gene segments (Fig. 3 A). Two D δ segments, two J δ segments, and five V δ sequences have thus far been reported (20). Of particular interest is whether the δ and α chains use the same pool of germline V gene segments. Use of the same V segment pool would not only indicate potential similarities in the specificity repertoire of α/β and γ/δ cells, but would indicate that the potential diversity of δ chains (and therefore of the γ/δ receptor) is quite large; there are estimated to be over 50 V α genes in the mouse (21). Of five V δ sequences reported by Chien et al. (14, 20), two are quite similar to the V α 7 gene, whereas the others are only distantly related in sequence to other known V gene sequences.

To evaluate the diversity of γ and δ chains used by murine γ/δ cells, we have analyzed a panel of five γ/δ receptor expressing hybridomas produced by fusion of a TCR β -loss variant of the tumor cell line BW5147 with an enriched population of γ/δ cells from the young adult thymus of C57BL/6 (B6) mice (22). A previous protein analysis of the receptor on four of these cell lines suggested that at least two electrophoretically distinct γ chains and at least three δ chains are represented (22). In addition, we have analyzed the enriched population of γ/δ cells with various δ probes to arrive at an estimate of the diversity of expressed V δ gene segments in the adult thymus.

Materials and Methods

Cell Lines. The DN7.1, DN12.1, DN7.3, and DN2.3 T hybridomas were described by Marusic et al. (22). The DN1.1 hybridoma was isolated in the same fusion (S. Marusic-Galesic, T. Saito, L. Tentori, J. Zuniga-Pflucker, D. H. Raulet, J. P. Allison, and A. M. Kruisbeek, manuscript submitted for publication). The T cell hybridomas were grown in RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum and 50 μ M 2-ME. The enriched population of thymic γ/δ cells was prepared as described (4) by culturing CD4⁺, CD8⁺ thymocytes (~5% γ/δ cells) from young B6 mice in a lymphokine cocktail for 3 d. The resultant population was analyzed by immunofluorescent staining and immunoprecipitation with anti-CD3 antibodies, and is estimated as 50–80% γ/δ -bearing, CD3⁺ cells.

cDNA Cloning. RNA was isolated from freshly harvested cells by lysis in guanidinium isothiocyanate (23). Poly(A)⁺ mRNA was purified by two cycles of oligo(dT) cellulose chromatography.

Double-stranded (ds) cDNA from DN 7.3, DN 7.1, and DN2.3 mRNAs was synthesized by the procedure of Gubler and Hoffman (24) using Moloney Leukemia Virus reverse transcriptase and RNase H. The dsDNA was treated with T4 DNA polymerase and Eco RI methylase before ligation of Eco RI linkers. The dsDNA was digested with Eco RI and size fractionated by agarose gel electrophoresis. dsDNA 1–4 kb in length was isolated by binding to glass beads (25) and ligated to λ gt 10. Recombinant phage were packaged using Gigapack (Stratagene Cloning Systems, San Diego, CA), and libraries were screened without amplification. The DN 7.3 library was probed with an oligonucleotide corresponding to the

C α sequence of Chien et al. (14) (5'CCAGCCTCCGGCCAAACCATCTG). The DN7.1 and DN2.3 libraries were probed with a 900 bp Eco RI fragment encoding C δ and 3' untranslated (3'UT) sequences, which was excised from a full-length δ 7.3 cDNA clone.

DNA was sequenced by a combination of chemical cleavage (26) and dideoxy chain termination on dsDNA using a modified form of T7 DNA polymerase (Sequenase; USB Biochemical Corp., Cleveland, OH). For sequence and Southern analysis of J δ 2 genomic sequences, two oligonucleotides corresponding to the 5' (5'CCCGACAGATGTTTTTGGAACTGGC) and 3' (5'GGGGCTCCACAAAGAGCTCTATGCC) halves of the J δ 2 coding region were synthesized.

Blot Hybridization. The filters were hybridized as described (27) with 3×10^7 dpm of each probe, labelled by random hexamer priming (28); filters were washed at 68°C with $2 \times$ SSC, 0.05% SDS, and exposed to preflashed Kodak XAR film.

Results

New V δ and J δ Gene Segments. To examine the δ chain diversity in the hybridomas, δ cDNA clones were isolated from three of the cell lines, DN7.3, DN7.1 and DN2.3 (Fig. 1). The deduced amino acid sequences of the 5' portions of these cDNA clones have several features common to other V regions, including numerous conserved residues (Fig. 1 B). One of the cDNAs, δ 7.1, is identical to the V_{M21} V region described by Chien et al. (20). Two of the cDNAs, δ 7.3 and δ 2.3 are novel V δ genes. Of the three cDNAs, two (V δ 7.1 and V δ 7.3) are only distantly related to known V α gene segments (<29 and 37% amino acid identity, respectively). The third cDNA, δ 2.3, is very similar to V α 4 gene segments, with 95.3% amino acid identity and 97% nucleotide identity to the TA65 V region reported by Arden et al. (29). This finding documents the functional expression of a V α -like gene as part of a δ chain on a γ/δ cell line.

Assuming that the NH₂ termini of the mature δ chains are as indicated in Fig. 1, the δ 7.1 and δ 7.3 cDNAs encode polypeptides of 29,966 and 30,477 daltons, respectively (note that the δ 2.3 cDNA does not extend to the NH₂ terminus). The sizes of the δ chains of DN7.1, DN7.3, and DN2.3 determined by SDS-PAGE analysis after enzymatic removal of NH₂-linked glycans, are 34, 35, and 36 kD, respectively (22). As has been noted previously, the discrepancies between the calculated molecular weights and determined molecular masses of δ chains may be due to O-linked glycosylation or other posttranslational modifications of the polypeptide, or may reflect aberrant gel mobilities of δ chains (14).

Each of the cDNAs include productive (in frame) V δ -D δ -J δ joints (Fig. 1 C). The V δ 7.3 gene segment is juxtaposed to J δ 1. V δ 2.3 is also rearranged to J δ 1; however this joining event results in the deletion of three amino acids encoded by the 5' end of J δ 1. In contrast, V δ 7.1 is juxtaposed to the J δ 2 gene segment, which is only moderately homologous to J δ 1. This is the first report of a functional rearrangement to J δ 2, indicating that the δ locus includes at least two functional J δ gene segments.

For further analysis of J δ 2, we cloned the germline J δ 2 gene segment. Because Southern hybridization analysis indicated that J δ 2 lies between J δ 1 and C δ (see below) we isolated genomic clones from a BALB/c liver library that hybridized with C δ and analyzed the region upstream of C δ for sequences that hybridized with an oligonucleotide probe corresponding to the J δ 2 sequence. The determined J δ 2 germline sequence (which is identical to that recently reported by Chien et al., [20]), and D δ and J δ 1 gene segment sequences reported by Chien et al. (20), were compared with δ cDNA clones to assess junctional diversity. This comparison reveals

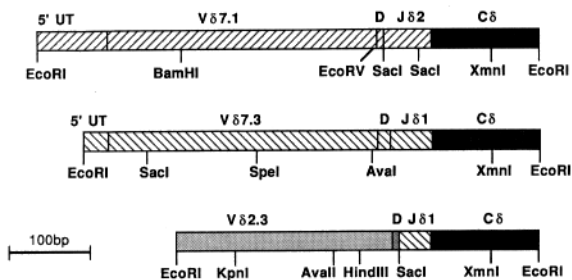
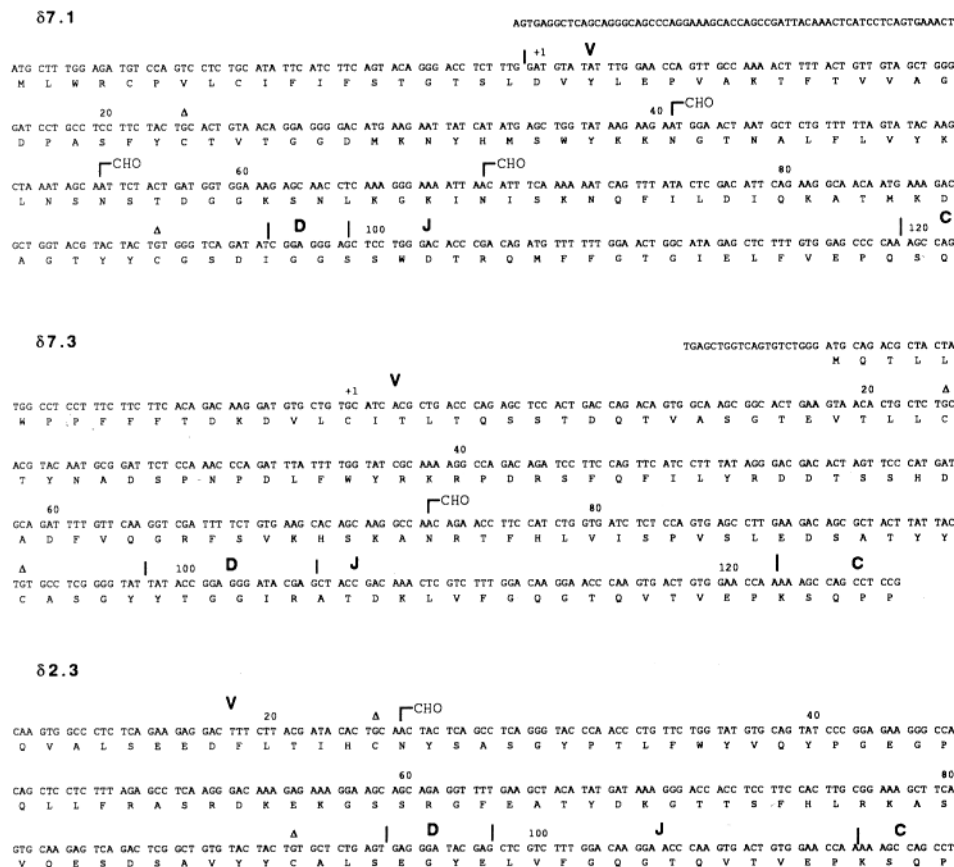
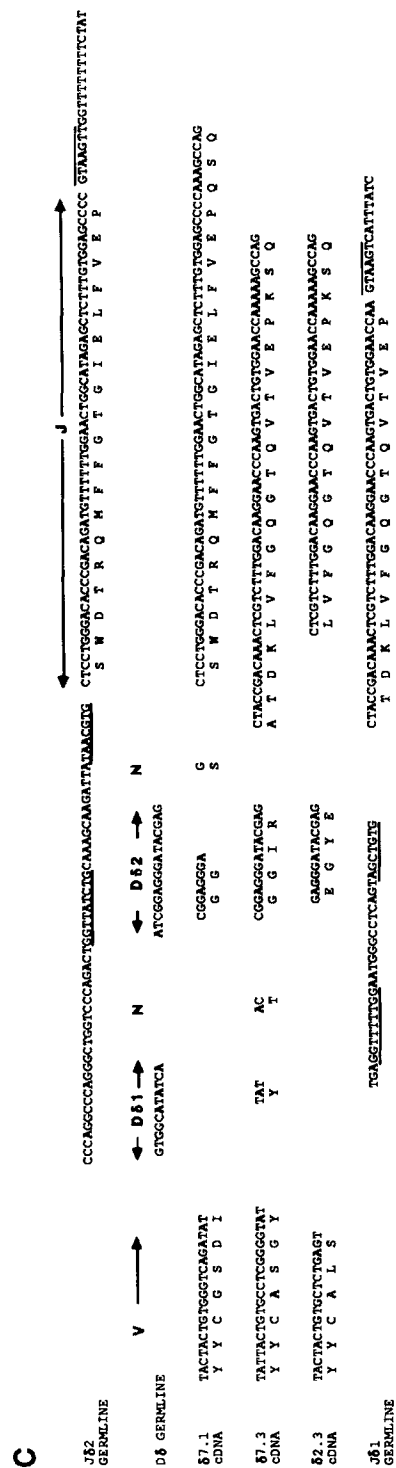
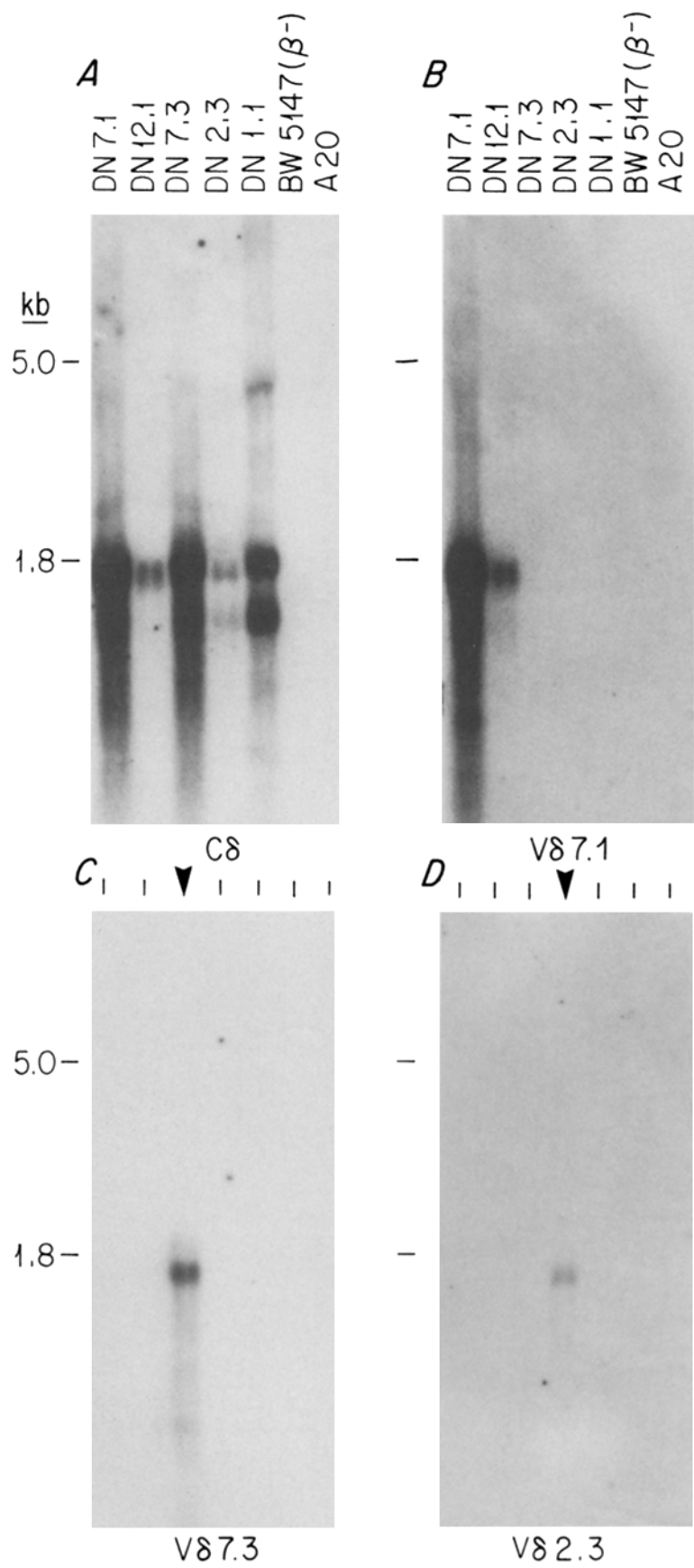
A**B**

FIGURE 1. (A) Restriction maps of 5' EcoRI fragments of $\delta 7.1$, $\delta 7.3$, and $\delta 2.3$ cDNAs indicating location of 5'UT, V, D, J, and C δ sequences. (B) DNA sequence and deduced amino acid sequences of $\delta 7.1$ and $\delta 7.3$, and $\delta 2.3$ cDNAs. Conserved cysteine residues thought to participate in disulfide bond formation are indicated by Δ . Conserved residues common to other V regions are numerous, including C(23), C(94), W(36), Y(37), Y(92), and Y(93). Potential carbohydrate addition sites (N-X-S/T) are indicated by CHO over the asparagine residue. The amino terminus of $\delta 7.1$ is indicated as +1 by comparison to protein sequence derived by Born et al. (19). The amino terminus of $\delta 7.3$ has been approximated by analogy to $\delta 7.1$.



Vst7.3 sequence is not derived from the germline *Vst7.3* gene (our unpublished data), and may therefore be derived from D81 as indicated. Heptamer and monomer recombination signals in J81 and J82 have been underlined. Splice donor sequences at the 3' side of J81 and J82 have been indicated by a line over the sequence.



that each of the cDNAs use D δ 2 gene segments. δ 7.3 may use D δ 1 sequences, since germline V δ 7.3 sequences do not account for the observed additional nucleotides (unpublished data).

Four Distinct δ Genes Are Expressed in a Panel of Five γ/δ T Hybridomas. Northern hybridization analysis with a C δ probe detected a transcript of \sim 1.8 kb in all five hybridomas, although DN12.1 and DN2.3 have considerably lower levels of this C δ transcript (Fig. 2). A smaller C δ transcript, possibly representing a DJC-transcript, was apparent in some of the lines. Neither transcript was detectable in BW5147, the fusion partner of the hybridomas. A V δ 7.3 probe detected a 1.8 kb transcript only in the DN7.3 hybridoma. Therefore, of the five hybridomas, only the DN7.3 cell line expresses detectable levels of a V δ 7.3 transcript.

A V δ 7.1 probe detected 1.8 kb transcripts in two cell lines (DN7.1 and DN12.1). The levels of this transcript are roughly concordant with the levels of the 1.8 kb C δ transcript in these cells. Hence the DN7.1 cell line and possibly the DN12.1 cell line express functional V δ 7.1 transcripts. A V δ 2.3 probe detects a transcript only in the DN2.3 cell line (Fig. 2).

To assess the δ rearrangements in the hybridomas, Southern hybridization analysis was performed with V δ and J δ probes (Fig. 3). A single band of 7.0 kb hybridized with the V δ 7.1 probe in an Eco RI digest of liver DNA, suggesting that this V segment represents a single-membered subfamily (Fig. 3 *B*). Both copies of V δ 7.1 are deleted in the BW5147 cell line. In the DN7.1 cell line, there are two rearrangements of the V δ 7.1 gene segment: to J δ 2 on one chromosome (a 4.5 kb Eco RI fragment) (Fig. 3 *B* and *C*) and to J δ 1 on the other (a 9.0 kb Eco RI fragment) (Fig. 3 *B* and *E*). Because the cDNA clone isolated from DN7.1 corresponded to a productive V δ 7.1-J δ 2C δ rearrangement, the 4.5 kb Eco RI fragment presumably encodes the expressed δ chain in the DN7.1 cell line. In the DN12.1 cell line the V δ 7.1 gene is also rearranged to J δ 2, detected as a 4.5 kb Eco RI fragment with both probes. The intensity of hybridization to this fragment compared with that in the DN7.1 hybridoma suggests that both chromosomes have undergone V δ 7.1-J δ 2 rearrangements. Both copies of J δ 1 are deleted in the DN12.1 cell line, as a result of the V δ -J δ 2 rearrangements. The data indicate that the expressed δ chain in the DN12.1 cell line is the product of a V δ 7.1-J δ 2 rearranged gene, consistent with the earlier observation that the δ chains of DN7.1 and DN12.1 are indistinguishable by nonequilibrium pH gradient electrophoretic analysis (29a). The independent origin of these two cell lines is indicated by the presence of presumably nonfunctional γ and δ rearrangements specific to each cell line (Figs. 3 and 4, see below).

A V δ 7.3 probe hybridized to a single 10.5 kb Eco RI fragment in B6 liver DNA suggesting that, like V δ 7.1, V δ 7.3 is a member of a single-membered V gene subfamily (Fig. 3 *D*). A rearranged 9.4 kb Eco RI fragment in the DN7.3 hybridoma hybridized with both the V δ 7.3 and J δ 1 probes, indicating that this rearranged gene

FIGURE 2. Expression of C δ and V δ gene sequences in RNA from DN hybridomas. 10 μ g of total RNA was electrophoresed on denaturing formaldehyde agarose (1.1%) gels. (A) Hybridization with C δ , a 900 bp Eco RI fragment from δ 7.3 cDNA containing C δ coding and 3'UT sequences. (B) Hybridization with V δ 7.1, a 240 bp Bam HI-Eco RV fragment from δ 7.1 cDNA. (C) Hybridization with V δ 7.3, a 370 bp Eco RI-Ava I fragment from δ 7.3 cDNA. (D) Hybridization with V δ 2.3, a 210 bp Eco RI-Hind III fragment from δ 2.3 cDNA.

A

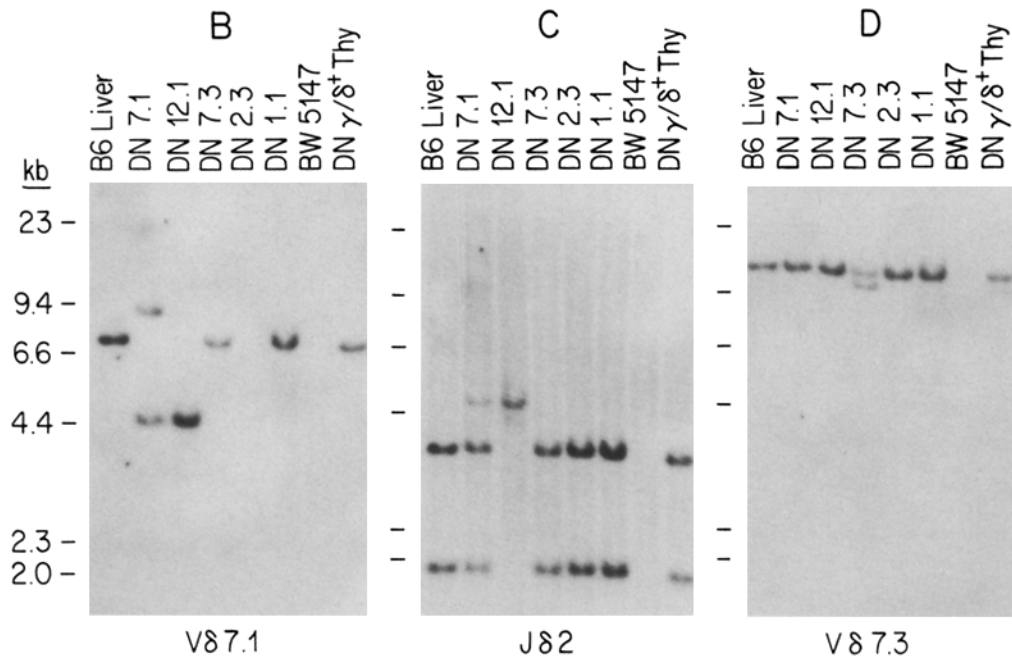
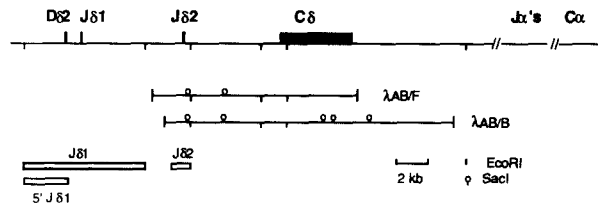
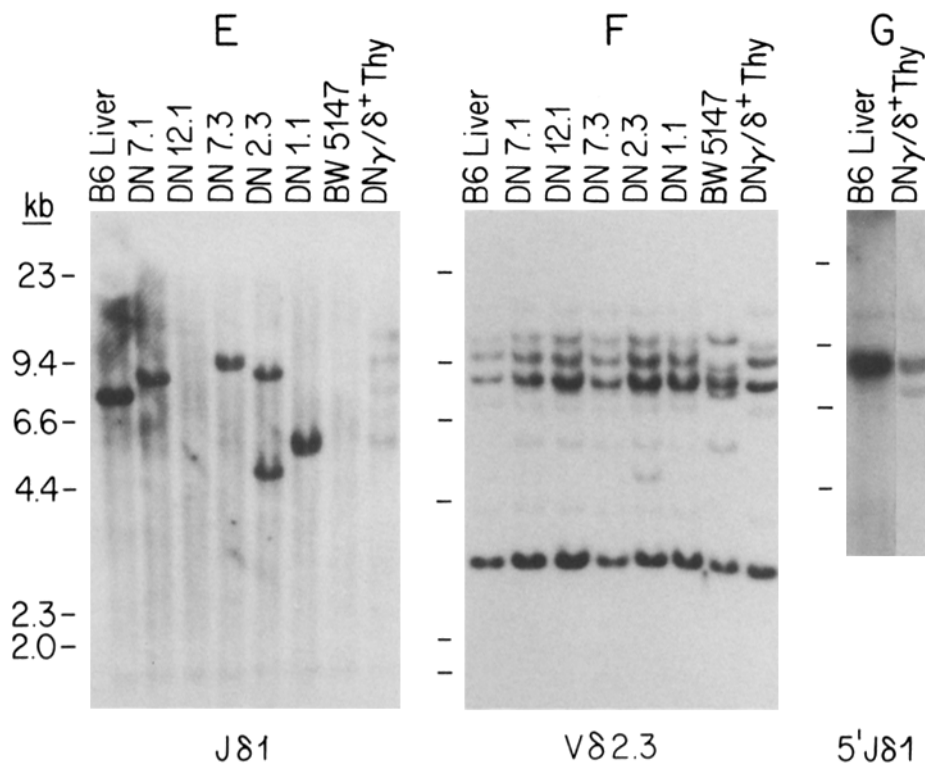


FIGURE 3. (A) Organization of the TCR δ chain locus in the BALB/c mouse indicating the location of J δ 1, J δ 2, and C δ gene segments (from Chien et al. [14, 20] and this study). A genomic library of BALB/c liver DNA (kindly provided by Dr. André Bernards, Whitehead Institute) was screened with a 900 bp Eco RI fragment containing C δ coding and 3'UT region sequences. Two phage clones, λ AB/F and λ AB/B, were isolated and mapped as shown. DNA probes isolated from genomic DNA and used for Southern hybridization are indicated (J δ 1, 5'J δ 1, and J δ 2, described below). (B-F) Southern analysis of V δ and J δ rearrangements in DNA from DN hybridomas and γ/δ -enriched double negative C57BL/6 thymocytes. All DNAs were digested with Eco RI, electrophoresed on 0.7% agarose gels and transferred to nitrocellulose. Hind III-digested λ DNA size markers are indicated as shown. (B) Hybridization with V δ 7.1 probe (see Fig. 2). (C) Hybridization with J δ 2 probe (a 1.2 kb Hind III-Eco RI fragment isolated from λ AB/F). The J δ 2 probe which is contained within a 2.85 kb Eco RI fragment in BALB/c DNA hybridizes to two Eco RI fragments in C57BL/6 DNA as shown here and similarly in Lindsten et al. (43). (D) Hybridization with V δ 7.3 probe (see Fig. 2). (E) Hybridization with J δ 1 probe (kindly provided by Dr. Astar Winoto, Whitehead Institute), a 7.5 Eco RI fragment isolated from C3H DNA. The bands in DN7.3 and DN1.1 are visible as doublets in the original autoradiograph. (F) Hybridization with V δ 2.3 probe (see Fig. 2). (G) Hybridization with 5' J δ 1, a 2.6 kb Eco RI-Hinc II fragment from the 5' side of the 7.5 kb J δ 1-containing Eco RI fragment.



represents the expressed δ gene. Rearrangements of the V δ 7.3 gene were not detected in any of the other hybridomas. An additional rearranged Eco RI fragment of 9.5 kb was detected in the DN7.3 cell line with the J δ 1 probe, presumably corresponding to a nonfunctional δ rearrangement.

Southern analysis using a V δ 2.3 probe detects seven Eco RI fragments in B6 liver DNA, presumably corresponding to multiple members of the V α 4 subfamily (Fig. 3 F). In the DN2.3 cell line, a rearranged band of 5.0 kb is detected with both V δ 2.3 and J δ 1 probes. DN2.3 also contains a second rearrangement (a 9 kb Eco RI fragment) detected with a J δ 1 probe.

The DN1.1 cell line expresses a γ/δ receptor, as demonstrated by immunoprecipitation analysis with an anti- δ chain antiserum (S. Marusic-Galesic, T. Saito, L. Tentori, J. Zuniga-Pflucker, D. H. Raulet, J. P. Allison, and A. M. Kruisbeek, manuscript submitted for publication). Two rearrangements of the J δ 1 gene segment were detected in this cell line, which are not detected with V δ 7.1, V δ 7.3, or V δ 2.3 gene segments (Fig. 3 E). The sizes of the rearranged fragments detected with a J δ 1 probe in DN1.1 (a doublet at \sim 6 kb) are different from those in the other cell lines, indicating that DN1.1 expresses a distinct V δ -J δ 1 rearranged gene. Therefore four distinct V δ genes are used in a panel of five γ/δ cell lines.

Three Different γ Chains Are Expressed in a Panel of Five γ/δ T Hybridomas. To determine the identity of the γ chains expressed by the hybridomas, Southern analysis of gene rearrangements and RNA analysis were performed. Interpretation of the

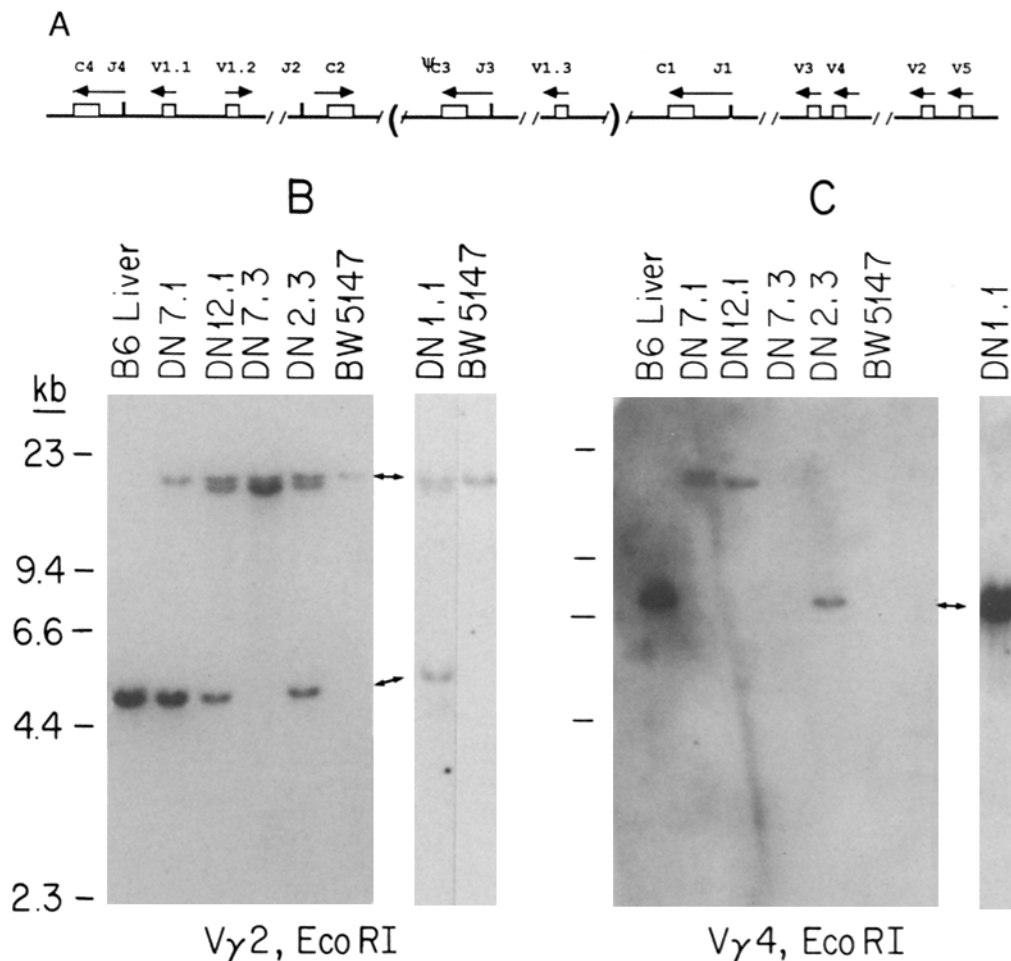
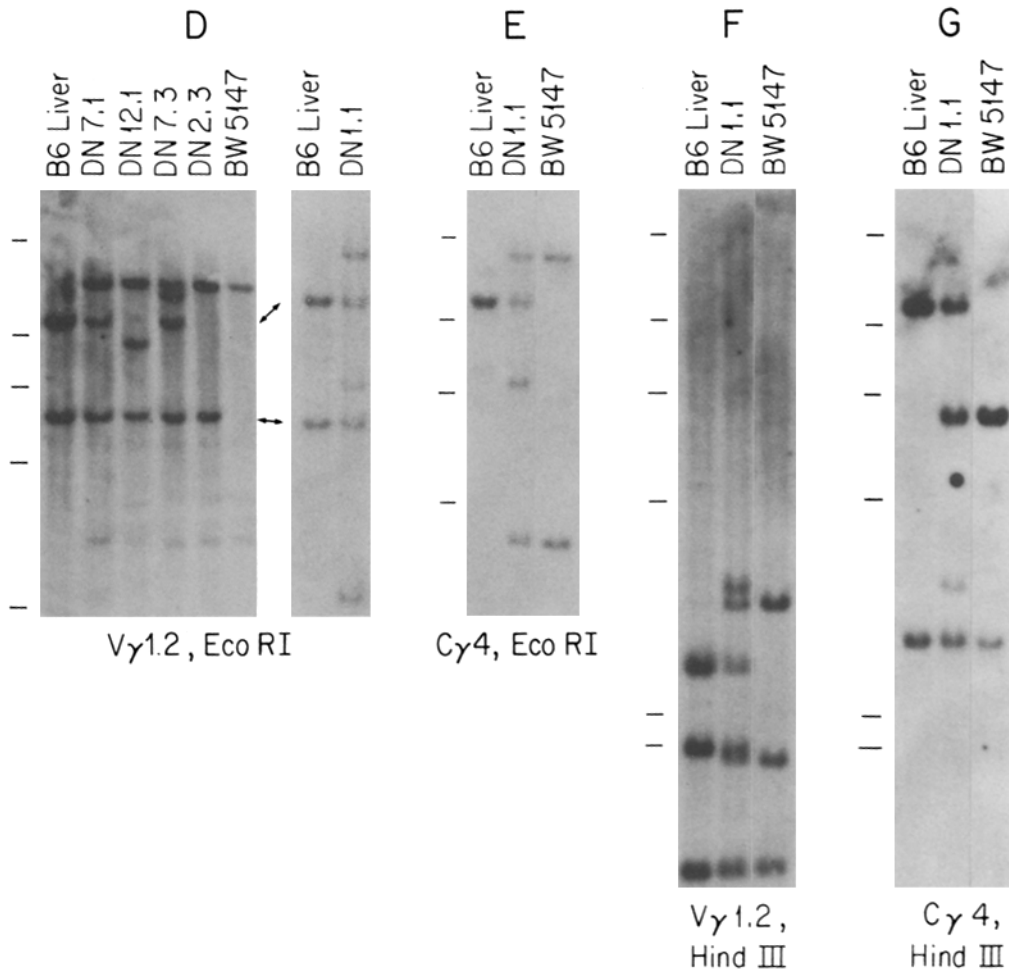


FIGURE 4. (A) Organization of TCR γ chain locus (based on refs. 9–11, 13). Relative location and transcriptional orientation of V γ and J γ C γ sequences are shown. The orientation of the V γ 1.3, J γ 3C γ 3 genes relative to the others has not been established. (B–G) Southern analysis of γ rearrangements in DNA from DN hybridomas. All DNAs were digested with Eco RI, electrophoresed on 0.7% agarose gels and transferred to nitrocellulose. Hind III-digested λ DNA size markers are indicated as shown. The V γ 2, V γ 4, and V γ 1.2 probes were those described in Garman et al. (10). The C γ 4 probe is a 1.6 kb Eco RI–Bam HI genomic fragment that includes the first exon of C γ 4 and ~700 bp of DNA in the J γ 4–C γ 4 intron. The following information is helpful in analysis of the blot hybridization data: (a) Donor B6 thymocyte-derived V γ 2–C γ 1C γ 1 rearrangements can be distinguished from the BW5147-derived V γ 2–J γ 1C γ 1 rearrangement due to a polymorphism between AKR (strain of origin BW5147) and B6 in an Eco RI site located 3' of the C γ 1 gene; the B6- and AKR-derived rearranged V γ 2–J γ 1C γ 1 genes reside on 17 and 19 kb Eco RI fragments, respectively (B). (b) Because V γ 4 and V γ 3 are closely linked on the same germline Eco RI fragment, the V γ 4 probe detects rearrangement of both genes; rearrangement of V γ 3 or V γ 4 to J γ 1 results in an 18 or 17 kb Eco RI fragment, respectively (C). (c) Donor B6-derived rearrangements to J γ 4 were detected in three cell lines, DN1.1 (G), DN12.1 and DN7.3 (not shown), by probing Hind III-digested DNAs with a C γ 4 probe. DN12.1 and DN7.3 both have V γ 1.1–J γ 4C γ 4 rearrangements (a 3 kb Hind III band). This rearrangement yields a 9.5 kb Eco RI fragment in DN12.1 which is visible by hybridization with V γ 1.2 (D). The 14 kb Eco RI band in DN7.3 is the result of V γ 1.1–J γ 4C γ 4 and V γ 1.2–J γ 2C γ 2 rearrangements on the same chromosome (V γ 1.1, V γ 1.2, and J γ 4C γ 4 are all on the same germline Eco RI fragment (9, 11).



In DN1.1, the rearrangement of V γ 1.3 to J γ 4C γ 4 yields a 6.6 kb Eco RI fragment and a 3.5 kb Hind III fragment (E and G), consistent with the location of germline Eco RI sites (9). For comparison, rearrangement of V γ 1.1 to J γ 4 yields a 9.5 kb Eco RI fragment (see above); based on the location of Eco RI sites in germline DNA, rearrangement of V γ 1.2 to J γ 4 would yield an 11.6 kb Eco RI fragment. (d) Hybridization with C γ probes (not shown) revealed no bands that cannot be accounted for by the rearrangements listed in Fig. 5.

Southern analysis is complicated by the fact that BW5147, the fusion partner, donates several rearranged γ genes to the hybridomas (13, 30) (Figs. 4 and 5): V γ 1.2-J γ 2C γ 2 (one allele), V γ 2-J γ 1C γ 1 (one allele), and V γ 5-J γ 4C γ 4 (one allele). The number of rearranged alleles of each type is based on the observation by Pelkonen et al. (13) that the V γ 5-J γ 4C γ 4 rearrangement on one of the two BW5147 chromosomes has deleted the intervening DNA (which includes the V1.1-V1.3, V2-4, and C1-C3 γ -gene segments). Previous studies indicate that both the V γ 1.2-J γ 2C γ 2 and V γ 5-J γ 4C γ 4 rearranged genes of BW5147 are nonproductive (13, 30).

| | REARRANGEMENTS | | | | | | | | RNA | | | | | | PROTEIN | | | ASSIGNMENT | |
|--------|----------------|-----------|---------|---------|---------|-----------|---------|---------|------------|------------------|----|----|----|----|--------------------|--------------|---------------|------------|-----------|
| | DONOR | | | | | | | | | | | | | | ANTI-C1,2 REACTIVE | Mr | | | |
| | BW | | | | | | | | C1, C2, C3 | V1.1, V1.2, V1.3 | V2 | V3 | V4 | C4 | | NO TREATMENT | N-GLYCOSYLASE | | |
| | V1.1-J4C4 | V1.3-J4C4 | V2-J1C1 | V3-J1C1 | V4-J1C1 | V1.2-J2C2 | V2-J1C1 | V5-J4C4 | | | | | | | | | | | |
| BW5147 | | | | | | | | | | | | | | | | | | | |
| DN7.1 | - | - | - | + | + | + | + | + | + | + | + | - | + | + | + | + | 34 | 31 | V4-J1C1 |
| DN12.1 | + | - | + | - | + | + | + | + | + | + | + | + | - | + | + | + | 34 | 31 | V4-J1C1 |
| DN7.3 | + | - | + | - | - | + | + | + | + | + | + | + | - | - | + | + | 35 | 32 | V2-J1C1 |
| DN2.3 | - | - | + | - | - | + | + | + | + | + | + | + | - | - | + | + | 35 | 32 | V2-J1C1 |
| DN1.1 | - | + | - | - | - | + | + | + | + | + | + | + | - | - | + | - | 38 | ND | V1.3-J4C4 |

FIGURE 5. Summary of γ gene rearrangements and transcripts present in the five γ/δ T hybridomas. The rearrangements are based largely on the data in Fig. 4. The V γ 1.1-J γ 4C4 rearrangements were assessed by Southern blot analysis of Hind III-digested DNAs, probed with C γ 4 and V γ 1.2 probes (not shown). The presence of the indicated transcripts were determined by ribonuclease protection assays of total RNA (not shown). A C γ 2 probe was used to detect C γ 2 and the crosshybridizing C γ 1 and C γ 3 transcripts, and a V γ 1.2 probe was used to detect V γ 1.1, V γ 1.2, and V γ 1.3 transcripts. Where a negative is indicated in the table, no band was visible even in overexposures of the autoradiograph. The protein relative molecular masses are from references 22 and 29a.

The γ chains of the DN7.3 and DN2.3 cell lines are encoded by V γ 2-J γ 1C γ 1 rearranged genes. B6-derived V γ 2-J γ 1C γ 1 rearrangements are present in both cell lines, and can be distinguished from the BW5147-derived V γ 2-J γ 1C γ 1 rearrangement (Fig. 4 B). Although other rearranged γ genes are present (Fig. 5), they cannot account for the properties of the γ chains on these cell lines, which are N-glycosylated (unlike the V γ 1.2-J γ 2C γ 2-encoded chain [8]) and react with an anti-C γ antiserum, which does not detect C γ 4 (22). Consistent with this assignment, the γ -chains of DN7.3 and DN2.3 are indistinguishable from each other by size, glycosylation pattern and isoelectric point (22, 29a). Because it is not known whether the BW5147-derived V γ 2-J γ 1C γ 1 rearranged gene is functional, it is not possible to definitively assign the γ chains in DN7.3 and DN2.3 as of B6-thymocyte origin. Of note in this context, however, is that the BW5147-derived V γ 2-J γ 1C γ 1 gene is not expressed as mRNA in BW5147, nor in a hybridoma, DN7.1, that uses a distinct γ gene (see below) and has no B6-donor-derived V γ 2 gene rearrangement (Fig. 5). Thus the BW5147 allele may be specifically inactivated in the hybridomas.

The γ chain of the DN7.1 cell line is encoded by a gene other than V γ 2, because this cell line lacks V γ 2 transcripts as well as donor-derived V γ 2 gene rearrangements. Instead, V γ 3-J γ 1C γ 1 and V γ 4-J γ 1C γ 1 rearranged genes are present in this cell line. Because the γ chains of DN7.1 and another cell line, DN12.1, are indistinguishable by SDS-PAGE and NEPHGE analyses, and DN12.1 has a rearranged V γ 4-J γ 1C γ 1 gene but no V γ 3-J γ 1C γ 1 gene, we conclude that the γ chains on both cell lines are encoded by V γ 4-J γ 1C γ 1 rearranged genes. Consistent with this assignment, the γ chains on both cells are N-glycosylated and react with the anti-C γ antiserum that is specific for C γ 1 and C γ 2 but not C γ 4. In addition, the size of the γ chains on these hybridomas (34 kD) is significantly less than that of the V γ 3-J γ 1C γ 1-encoded

γ chain (42 kD) expressed by a dendritic epidermal cell line (31), probably due in part to the presence of an acceptor site for *N*-glycosylation in V γ 3 that is absent in V γ 4 (10). Furthermore, the DN12.1 and DN7.1 γ chains are smaller than the V γ 2-J γ 1C γ 1 chains expressed by DN7.3 and DN2.3 (35 kD).

The DN1.1 cell line expresses yet a third γ chain, because it has no donor-derived V γ 2, V γ 3, or V γ 4 rearrangements. The putative γ chain of DN1.1 is not reactive with anti-C γ 1,2 antiserum (S. Marusic-Galesic, T. Saito, L. Tentori, J. Zuniga-Pflucker, D. H. Raulet, J. P. Allison, and A. M. Kruisbeek, manuscript submitted for publication), and therefore is likely a product of the C γ 4 gene. Consistent with this assignment, a heretofore unreported B6 donor-derived rearrangement of the C γ 4 gene on a 6.6 kb fragment in an Eco RI digest (Fig. 4 E) and a 3.5 kb fragment in a Hind III digest (Fig. 4 G) was present in this cell line. Because a V γ 1.2 probe hybridized to fragments of identical size in both digests (Fig. 4 D and F), we conclude that one of the three-members of the V γ 1 gene subfamily has rearranged to J γ 4 in the DN1.1 cell line. The sizes of the rearranged fragments are those expected from rearrangement of V γ 1.3 to J γ 4, and are inconsistent with rearrangements of either V γ 1.2 or V γ 1.1 to J γ 4 (Fig. 4). Although a BW5147-derived V γ 5-J γ 4C γ 4 rearranged gene is also present in this cell line, an earlier study found that this gene is nonfunctional (13). Donor-derived V γ 5-J γ 4C γ 4 rearrangements are apparently not present in DN1.1, because a donor-derived V γ 1.2 gene, in germline configuration, is retained. Rearrangement to J γ 4 of both V γ 1.3 and V γ 5 in the same cell should delete both intervening V γ 1.2 genes. Therefore, this cell line contains only one rearrangement involving J γ 4C γ 4 that can encode the expressed γ chain: V γ 1.3-J γ 4C γ 4 (B6 thymocyte derived).

As expected, each hybridoma expressed the transcript corresponding to the gene determined to be functional in the analysis described above (summarized in Fig. 5). In addition, however, each hybridoma expressed other γ transcripts, which may be nonfunctional. The expression of nonproductive γ transcripts in T cells has been frequently observed (12, 30, 32–34). Taken together, our data suggest that three distinct γ chains encoded by V γ 2-J γ 1C γ 1, V γ 4-J γ 1C γ 1, and V γ 1.3-J γ 4C γ 4 rearranged genes, are represented among five γ/δ T hybridomas.

Restricted Use of δ and γ Chains in an Enriched Population of Thymic γ/δ Cells. To determine the approximate frequency of δ rearrangements in thymic γ/δ cells, we analyzed the rearrangements present in DNA from an enriched population of such cells (50–80% γ/δ cells) isolated by short-term (3 d) culture in a lymphokine cocktail (4). Because most of these cells express a γ/δ receptor, quantitation of various δ rearrangements represents an approach to determining the usage and diversity of V δ genes in the population. Detection of such rearrangements by Southern blot analysis suffers from the fact that rearrangements that are relatively rare in the population will escape detection; if there are many such rearrangements, a good deal of the diversity of δ gene rearrangements will be overlooked. On the other hand, if most of the alleles can be quantitatively accounted for by visible rearrangements, some estimate can be made of the diversity of δ rearrangements in the population. Although Southern analysis does not allow determination as to whether a rearrangement is functional (in frame), the fact that the majority of cells in the population express a δ chain means that most of the cells have at least one functional rearrangement (see below).

Five distinct rearrangements of J δ 1 were detected in the population with a J δ 1 probe (Fig. 3 *E*). One fragment of 9.4 kb corresponds to a rearrangement of V δ 7.3 to J δ 1, as shown by hybridization with a V δ 7.3 probe (Fig. 3 *D*). Two predominant \sim 6 kb rearrangements in the population correspond in size to rearrangements in the DN1.1 cell line. One of these corresponds to a D δ 1-J δ 1 rearrangement, based on our recent finding that this band hybridizes with a 5' D δ 1 probe (not shown). The other 6 kb fragment presumably corresponds to the productively rearranged V δ gene in DN1.1, suggesting that this V gene is commonly rearranged by cells in the population. Of the two remaining rearranged bands detected in the population with a J δ 1 probe (\sim 6.6 and 13 kb), the 6.6 kb band represents a nonfunctional D δ 2-J δ 1 rearrangement; we found that a 2.5 kb Eco RI-Hinc II genomic DNA fragment that includes sequences 5' of D δ 2 in germline DNA hybridizes to the 6.6 kb Eco RI fragment in DNA from the enriched population (Fig. 3 *F*). Because D δ 2 is 0.9 kb upstream of J δ 1 in germline DNA (20), the size of this rearranged fragment is consistent with a D δ 2-J δ 1 rearrangement. Significantly, none of the visible rearranged bands correspond to rearrangements of V δ 7.1 and V δ 2.3, as revealed by hybridization with these probes (Fig. 3 *B* and *F*).

To quantitate the visible rearrangements, we reprobbed the Southern shown in Fig. 3 *E* with a single-copy reference probe (a murine IL-6 cDNA), and subjected the autoradiographs to densitometric analysis (data not shown). By comparing the lanes containing liver DNA with the lanes containing γ/δ cell DNA, and normalizing for amount of DNA by reference to the single-copy probe, we found that the sum of DNA present in visible J δ 1 rearrangements corresponds to \sim 45% of the potential δ alleles in the population. It was therefore important to determine whether the remaining δ alleles are present as diverse, undetectable rearrangements or are deleted from the cells. A replicate filter was therefore hybridized with a mixture of a C δ cDNA probe and the IL-6 reference probe. Because the restriction enzyme used for these Southern blots, Eco RI, cuts between C δ and both J δ segments, the C δ alleles should not be obscured by rearrangements. Analysis of the autoradiographs by densitometry (data not shown) revealed that \sim 50% of the δ alleles are deleted from the population, perhaps by deletional rearrangement of V α gene segments to J α s (14). The data indicate that nearly all of the δ rearrangements in the population can be accounted for by the visible J δ 1 rearrangements described above. Because half the δ alleles are deleted, and most cells in the population express a δ chain, it follows that many or most of the rearrangements observed must be functional. The limited number of rearrangements observed indicate that most thymic γ/δ cells use one of a few V δ genes.

One commonly rearranged V δ gene is V δ 7.3. Densitometric analysis of Southern blots hybridized with J δ 1 and V δ 7.3 revealed that V δ 7.3 to J δ 1 rearrangements represent \sim 16–30% of the rearranged δ genes in the population. Since half the alleles are deleted from the population, these data suggest that a significant fraction of the cells express a V δ 7.3-J δ 1C δ encoded δ chain.

Rearrangements of the J δ 2 gene were undetectable in DNA from the thymic γ/δ cell population (Fig. 3 *C*). Densitometric analysis of the autoradiograph, in comparison with the same blot reprobbed with the single-copy reference probe (data not shown), revealed that the unrearranged J δ 2 bands account for the large majority (\sim 90%) of the δ alleles still present in the population, as expected from our finding (above)

that most of these δ alleles have undergone rearrangement to J δ 1 or are in germline configuration. These data suggest that J δ 2 rearrangements are rare in the population.

Taken together, our results suggest that most cells in the adult thymic γ/δ cell population express one of a few V δ genes rearranged to J δ 1. The abundance of the V δ 7.3-J δ 1 rearrangement in the population (16–30% of the δ alleles) suggests that this gene segment is one of the commonly expressed V δ gene segments; in contrast, the V δ 7.1 and V δ 2.3 gene segments appear to be only rarely used. Separate studies (34a, 34b) have found that the large majority of cells in the population express a γ chain encoded by a V γ 2-J γ 1C γ 1 rearranged gene. Therefore, most cells in this population appear to use receptors composed of a small subset of the available γ and δ subunits.

Nevertheless, rare cells in the population express other V δ -J δ and V γ -J γ genes, as indicated by the isolation of the DN7.1, DN12.1, and DN2.3 T hybridomas from the same population. We have not determined why the panel of hybridomas is apparently not numerically representative of the parent population, although it is possible that the short-term culture of the cells in the lymphokine cocktail before fusion preferentially stimulates a subset of cells, thus enhancing their fusion efficiency.

Discussion

Restricted Use of V γ and V δ Genes. Earlier studies have shown that the potential diversity of the γ chain in the mouse is relatively limited, with only seven (known) V gene segments, three functional J segments, and no known D segments (9–13). While there is clear evidence of junctional diversity created at V γ -J γ joints, presumably due in part to the addition of random nucleotides (*N* regions), the extent of *N* diversity in γ chains may be relatively limited (9–12). Moreover, γ gene use in the adult thymic population of γ/δ cells is strikingly nonrandom, with most cells expressing the product of a single rearranged gene, V γ 2-J γ 1C γ 1 (34a, 34b). Interestingly, γ gene use may depend on anatomical site and developmental stage, since protein and mRNA expression studies suggest that fetal thymocytes and the peripheral dendritic epidermal cells (DEC) commonly express γ chains other than V γ 2-J γ 1C γ 1 (10, 12, 31).

In this report we identify the rearranged genes that encode three different γ chains that are represented in a panel of five γ/δ T hybridomas: V γ 2-J γ 1C γ 1, V γ 4-J γ 1C γ 1, and V γ 1.3-J γ 4C γ 4. Expression of the two latter rearranged genes as γ polypeptides has not been previously reported. In concert with the analysis of the population, these data reveal a situation in which most adult thymic γ/δ cells express one γ chain, although a minor set of cells expressing other γ chains is present in the population. This situation is in contrast to that of the V α and V β chains of the α/β TCR, where predominant V gene use is not generally observed (35, 36). It seems unlikely that the predominant usage of V γ 2 by thymic γ/δ cells is due to preferential rearrangement of the V γ 2 gene in early T cells (as has been argued for the predominant usage of J_H-proximal V_H gene segments in pre-B cells [37] because fetal thymocytes preferentially rearrange the J γ 1-proximal V γ 3 and V γ 4 genes (10).

The potential diversity of δ genes is still under investigation. The δ locus includes at least two D δ segments and two J δ segments (20, and this report). A critical issue concerns the number of V δ gene segments, and whether V α and V δ gene segments are overlapping sets. In this report we provide evidence that four different V δ chains

are represented in a panel of five hybridomas. The deduced amino acid sequences of two of these V δ chains are very different than those of any known V α chain. Although the sequences are slightly more similar to V α sequences than to V sequences of the β or γ families, they differ from V α sequences about as much as most V α subfamilies differ from each other. A third V δ sequence (82.3), however, is very similar to a member of the V α 4 subfamily. Thus, including the earlier results of Chien et al. (14, 20), the sequences of four of six V δ subfamilies analyzed are very different from those of the known V α genes. Two of the genes show considerable homology to known V α gene segments, although neither are identical. These findings raise the possibility that V δ genes and V α genes are largely nonoverlapping sets. It remains to be determined whether this differential use of V genes is imposed by regulation of the allowable gene rearrangements to J δ s vs. J α s, by selection for chains able to pair with a given partner (β or γ) chain, or by selection of cells bearing particular receptors, among other possibilities.

In the present analysis, we have not attempted to estimate the total number of potential V δ genes. This number may be quite large, particularly if other V α genes are used for δ rearrangements. Nonetheless, Southern analysis of the thymic γ/δ population with J δ probes revealed several predominant rearrangements, suggesting that, like V γ genes, relatively few V δ genes are expressed by the majority of cells in this population. In the previous analyses of δ diversity (20), the majority of cells in the populations studied did not express a surface γ/δ receptor.

The V δ 7.3-J δ 1 rearrangement was a predominant rearrangement in the population, suggesting that V δ 7.3 is a commonly used V δ gene in thymic γ/δ cells. Considering the receptor as a whole, these results suggest that a V γ 2-J γ 1C γ 1/V δ 7.3-J δ 1C δ receptor, like the one expressed by the DN7.3 cell line, is one of a few dominant γ/δ receptors expressed in this population. In contrast to the expression of α/β receptors in peripheral T cells, the γ/δ receptors expressed in this population are dominated by those using one or a few V γ and V δ chains, respectively. A similar situation may exist in humans, since the diversity of expressed human V δ genes may also be limited (15).

Nonrandom Association of V γ and V δ Gene Segments. A striking finding of the present studies is that there may be nonrandom pairing of V γ and V δ chains. Both T hybridomas we analyzed that express a V γ 4-J γ 1C γ 1 chain also express a V δ 7.1-J δ 2C δ chain. In addition, the protein sequencing studies of Born et al. (19) have shown that a fetal T hybridoma that expresses V γ 4 expresses a δ chain that may be identical to V δ 7.1 (based on the identity of their peptide sequences with deduced peptide sequences of V δ 7.1). Considering that V δ 7.1 and J δ 2 rearrangements are rare in the adult thymic γ/δ cell population (as shown in this study) as are V γ 4 rearrangements (34b), the association of V δ 7.1-J δ 2C δ and V γ 4-J γ 1C γ 1 expression is unlikely to be coincidental. Considering the earlier finding that V γ 4 transcripts (10) and V δ 7.1 rearrangements (V $_{M21}$ [20]) are most abundant in fetal thymocytes, it is possible that cells expressing V γ 4/V δ 7.1 correspond to a fetal type that remain in small numbers in the adult thymus.

Another example of preferential pairing is suggested by the finding that most cells in the population bear a V γ 2-J γ 1C γ 1 chain (34a, 34b) and many of these cells may bear a V δ 7.3-J δ 1C δ chain, based on the abundance of the corresponding rearrangement. Because expression of both of these genes is common, the preferential pairing

may be coincidental. In any case, our data do not distinguish whether these associations are due to structural constraints on chain pairing, cellular selection, or reflect the developmental origin of the cells.

Function of the γ/δ Receptor. Our studies have not determined the extent of V δ -D δ -J δ junctional diversity, and therefore it is not possible to estimate the number of different receptor sequences that could be generated with limited V γ and V δ gene usage; this number may be quite large. The significant combinatorial and junctional diversity observed in γ and δ chains may be more consistent with a role for γ/δ receptors in recognition of foreign antigens than in recognition of predictable structures such as hormones or invariant cell surface structures. However, the limited use of V γ and V δ genes, and the apparent nonrandom pairing of V γ and V δ chains may indicate that important components of receptor chains that contribute to specificity (i.e., complementarity-determining regions 1 and 2, by analogy to immunoglobulins) are relatively nondiverse in the γ/δ receptors expressed in the adult thymus. Further studies will be required to assess γ/δ receptor diversity at other stages in ontogeny and in other tissues.

It is useful to consider the relatively limited diversity of expressed V γ and V δ genes in the context of a recent report demonstrating alloreactive γ/δ cells specific for an MHC-linked antigen (38). The target class I antigen was shown to be encoded by a gene in the H-2-D or linked Qa/Tla regions. The previous finding that alloreactivity to H-2 antigens is a common feature of T cells specific for foreign antigens plus self-H-2 (39, 40) raises the possibility that γ/δ cells also display restricted recognition of foreign antigens. The limited diversity of expressed V γ and V δ genes might then be partly explained by limited variability of the restricting elements. It is therefore attractive to speculate that Qa/Tla-encoded class I molecules (or a subset of them), which show relatively limited polymorphism, might serve as restricting elements for recognition of foreign antigens by at least some γ/δ T cells; according to this idea, the diversity of foreign antigens recognized by γ/δ cells may be related to the extent of junctional diversity in the chains. In support of the notion that Qa/Tla class I molecules may be restricting elements are their similarities in sequence to H-2 class I molecules in the $\alpha 1$ and $\alpha 2$ domains (reviewed in 41); these regions of H-2 class I molecules are believed to be involved in binding peptides of foreign antigens (42). A separate T cell receptor involved in restricted recognition might have evolved to deal with a special class of antigens.

Elliott et al. (44) recently analyzed a panel of δ cDNA clones from adult double negative thymocytes. They find extensive junctional diversity in δ chains, due to potential for V-D-D-J joining and N-region diversity at each of the junctions. The V $\delta 7.3$ sequence corresponds to their V $\delta 5$ sequence, which they find to be frequently expressed by adult double negative thymocytes.

Summary

Previous studies have indicated that the diversity of γ genes expressed by γ/δ -bearing murine T cells is limited, but comparable information concerning the expressed diversity of δ genes is lacking. In this study, we have investigated the rearrangement and expression of δ and γ genes in T cell hybridomas that express γ/δ T cell receptors. Three productive δ chain cDNA clones were isolated ($\delta 7.3$, $\delta 7.1$,

and $\delta 2.3$) that encode new variable region sequences. Two of the δ cDNAs differ significantly from those observed in the $V\alpha$ repertoire. In addition, one cDNA expressed a new J δ region (J $\delta 2$), which was localized between J $\delta 1$ and C δ genes. Using these and other δ gene probes and γ gene probes, we found that five independent hybridomas expressed four different V δ s and three different V γ s. However, analysis of an enriched population of γ/δ -expressing cells from the adult thymus suggests that only a few V δ genes and one V γ gene are used by the majority of the cells. These results suggest that important components of receptor chain that contribute to specificity (i.e., the germline V gene sequences) are relatively nondiverse in the thymic γ/δ population.

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